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The Role of the CD4 Receptor versus HIV Coreceptors in Envelope-Mediated Apoptosis in Peripheral Blood Mononuclear Cells

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We examined the role of CD4, CXCR4, and CCR5 in HIV envelope-mediated apoptosis by measuring the response of activated PBMCs to recombinant envelope proteins derived from CXCR4- and CCR5-utilizing viruses. Apoptosis of T cells was assessed by annexin-V staining and TdT-mediated dUTP-biotin nick-end labeling. Treatment of CCR5Δ32 homozygote PBMCs with a CCR5-specific envelope induced apoptosis in T cells, demonstrating that envelope–CD4 interactions are sufficient to induce apoptosis. However, a CXCR4-specific envelope induced higher levels of apoptosis than a CCR5-specific envelope, suggesting that envelope-mediated apoptosis can be enhanced by envelope–CXCR4 interactions. We conclude that envelope can induce apoptosis in T cells independently of the coreceptor specificity of a given envelope, or the expression profile of CXCR4 or CCR5 on a target cell. However, envelope–coreceptor interactions, and in particular, envelope–CXCR4 interactions, can contribute to this process.

Key Words: CCR5; CXCR4; retrovirus; gp120; TUNEL; annexin-V; T cell.

INTRODUCTION

The depletion of $CD4^+$ T lymphocytes is a central pathogenic feature of HIV-1 infection and is largely responsible for the profound immunodeficiency that is characteristic of late stages of HIV disease (Fauci, 1996; Mossman *et al.,* 1996). Although the mechanisms leading to $CD4^+$ T cell decline remain to be elucidated, it is generally agreed that cytolysis resulting from direct infection (Ho *et al.,* 1995) and enhanced rates of apoptosis (Badley *et al.,* 1997; Groux *et al.,* 1992; Monte *et al.,* 1992) contribute to this process. Paradoxically, although $CD4^+$ T lymphocytes represent the principal target of infection, relatively few $CD4^+$ T lymphocytes are infected (Biberfeld *et al.,* 1986), even in the presence of clinically high viral loads. Thus, additional mechanisms associated with viral replication but independent of direct infection are also believed to contribute to $CD4^+$ T cell killing. Several virally encoded proteins, including Tat (McCloskey *et al.,* 1997) and Vpr (Stewart *et al.,* 2000), have been implicated in HIV-mediated apoptosis. In addition, numerous studies have demonstrated that the viral envelope protein enhances the rate of apoptosis in activated $CD4^+$ T cells (Chirmule and Pahwa, 1996; Cicala *et al.,* 2000; Finkel *et al.,* 1995; Herbein *et al.,* 1998; Ullrich *et al.,* 2000). Thus, envelope proteins, present either as soluble proteins or on the surface of infectious and/or defective viral particles, have the potential to contribute to $CD4^+$ T cell depletion *in vivo.* This potential is underscored by the presence of high concentrations of envelope protein in lymphoid tissues (Sunila *et al.,* 1997).

Envelope-mediated apoptosis of T cells is a consequence of envelope-mediated signal transduction. In this regard a number of laboratories have demonstrated that envelope proteins transduce signals through the CD4 receptor as well as through the fusion coreceptors, CCR5 and CXCR4 (Cicala *et al.,* 1999; Davis *et al.,* 1997; Hivroz *et al.,* 1993; Popik *et al.,* 1998; Tamma *et al.,* 1997). Envelope-mediated signal transduction through CD4 results in the phosphorylation of p56-Lck (Hivroz *et al.,* 1993), while envelope signals delivered through CCR5 and CXCR4 result in responses that mimic chemokine-mediated signal transduction (Bacon *et al.,* 1996; Cicala *et al.,* 1999; Davis *et al.,* 1997; Ganju *et al.,* 1998). Most notably, envelope engagement of CCR5 results in chemotaxis (Weissman *et al.,* 1997), calcium mobilization (Arthos *et al.,* 2000), and the phosphorylation of several substrates including PYK-2 (Davis *et al.,* 1997), FAK (Cicala *et al.,* 1999), ZAP-70 (Tamma *et al.,* 1997), and CCR5 (Cicala *et al.,* 1999).

Although envelope can signal through CD4, CCR5, and CXCR4, it is unclear which of these signals participates in envelope-mediated apoptosis of primary T cells. Prior to the identification of CCR5 and CXCR4 as HIV corecep-

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tors, Finkel and colleagues demonstrated that crosslinking the CD4 receptor with monoclonal antibodies increased rates of apoptosis in activated $CD4^+$ T cells (Finkel *et al.,* 1995). Similarly, Berndt and colleagues demonstrated that monoclonal antibody (mAb)-mediated CD4 crosslinking induces apoptosis, but only by CD4 mAbs that recognize the gp120 binding site on CD4 (Berndt *et al.,* 1998). These observations suggest that CD4 engagement by gp120 alone could account for envelope-mediated apoptosis. A number of studies have demonstrated that envelope–CXCR4 interactions can induce apoptosis (Berndt *et al.,* 1998; Biard-Piechaczyk *et al.,* 2000; Colamussi *et al.,* 2000; Hesselgesser *et al.,* 1998; Kaul and Lipton, 1999; Ohagen *et al.,* 1999; Ullrich *et al.,* 2000; Zheng *et al.,* 1999). However, the role of CXCR4 signaling in apoptosis is unclear. Some researchers have found that SDF-1α induces apoptosis (Colamussi *et al.,* 2000), while others have found that it protects cells from apoptosis (Blanco *et al.,* 1999). Unlike CD4 and CXCR4, envelope–CCR5 interactions have not yet been implicated in envelope-mediated apoptosis.

The potential role of CXCR4, but not CCR5, in apoptosis is noteworthy in light of the differential effects of CXCR4 (X4) vs CCR5 (R5) viruses *in vivo.* R5 viruses are preferentially transmitted and predominate during the early stages of disease (van't Wout *et al.,* 1994). However, in some individuals the viral quasispecies evolves to a predominantly X4-utilizing phenotype. These individuals typically experience a more rapid decline in $CD4^+$ T cells and an increased rate of disease progression (Cheng-Mayer *et al.,* 1988; Tersmette *et al.,* 1989a,b). Thus, the acquisition of CXCR4 specificity is correlated with a more cytopathic virus and implies that envelope–CXCR4 interactions might be intrinsically more cytopathic to target cells than envelope–CCR5 interactions. However, in a series of reports, Grivel and colleagues, utilizing explanted lymphoid tissue, have convincingly demonstrated that, in the context of active replication, R5 and X4 viruses are similarly cytopathic, but that X4 viruses deplete $CD4^+$ T cells to a greater extent because the range of target cells that express CXCR4 is much greater (Grivel *et al.,* 2000a,b; Grivel and Margolis, 1999). Thus, it remains unclear whether envelope–CXCR4 engagement induces apoptosis to a greater degree than envelope–CCR5 engagement.

In this study we have compared the capacity of primary X4 vs R5 specific envelopes to induce apoptosis in activated T cells. In addition, we have dissected the role of envelope-mediated CD4 signaling vs chemokine-receptor signaling in envelope-mediated apoptosis.

RESULTS

Induction of apoptosis in peripheral blood T cells; comparison of a primary R5 vs X4 envelope

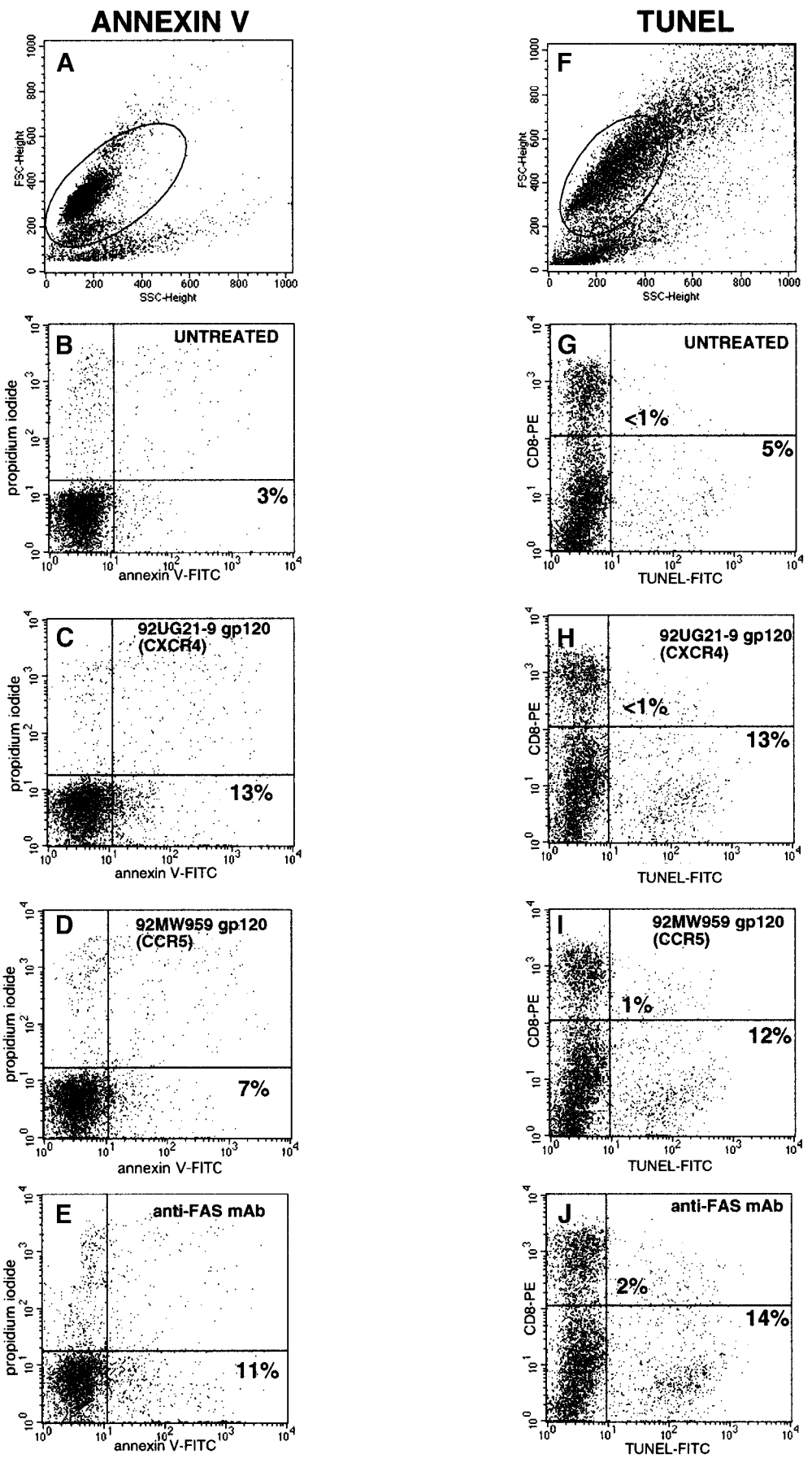
To quantitatively compare the capacity of R5 and X4 envelopes to induce apoptosis in activated peripheral blood mononuclear cells (PBMCs), freshly isolated PBMCs were activated with OKT3/IL2 for 3 days prior to treatment with envelope. We employed two independent assays. Increased exposure of phosphatidyl-serine on the outer surface of the cell membrane is one of the earliest events associated with the commitment of a cell to apoptosis and can be measured flow cytometrically with fluorescein-conjugated annexin-V (Bossy-Wetzel and Green, 2000). Degradation of chromosomal DNA occurs at the end of programmed cell death and can be measured flow cytometrically via TdT-mediated dUTP-FITC nick-end labeling (TUNEL) (Carbonari *et al.,* 1995). We compared two recombinant gp120s derived from primary viral isolates. 92MW959 gp120 was derived from an isolate that uses CCR5 exclusively, while 92UG21-9 was derived from an isolate that utilizes CXCR4 exclusively (Moore *et al.,* 1997).

We first compared the capacity of the X4 and R5 envelopes to induce apoptosis using the annexin-V assay. Five hours after envelope treatment, PBMC cultures were analyzed for annexin-V and propidium iodide (PI) staining. Cells staining positive for annexin-V and negative for PI were considered in the early stages of programmed cell death. The X4-specific envelope increased annexin-V reactivity fourfold (Fig. 1C). Utilizing the same donor PBMCs, the increase in apoptosis in response to the R5 envelope was twofold (Fig. 1D). In addition, fresh PBMCs were treated with the same recombinant envelopes and analyzed by TUNEL 65 h posttreatment (Figs. 1F–1J). TUNEL reactivity increased by more than twofold in all donors in response to the X4 envelope. The increase in TUNEL positivity resided within the population that excluded the CD8-bright lymphocyte population. Of note the CD8-bright cells failed to apoptose in the presence of the anti-FAS mAb, CH11, even though this population expresses CD95 (data not shown), indicating that in the culture system we employed, this population appeared resistant to apoptosis. Hence, in subsequent experiments we did not specifically address the levels of apoptosis in CD8-bright cells. We cannot exclude the possibility that populations other than $CD4^+$ T cells undergo apoptosis in response to envelope, and the effects of envelope on these subsets is currently under investigation (J. Arthos and C. Cicala, unpublished results).

In summary, both the R5 and X4 envelopes demonstrated the capacity to induce apoptosis in T cell lymphocytes. However the X4-specific envelope induced apoptosis to a greater degree, suggesting that signaling through CXCR4 may more effectively contribute to the induction of apoptosis than signaling through CCR5.

R5 envelopes induce apoptosis in PBMCs derived from a CCR5Δ32 homozygous individual

We next asked whether envelope–CD4 interactions in the absence of envelope–coreceptor interactions can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induce apoptosis in $CD4^+$ T cells. To address this issue, we employed freshly isolated PBMCs derived from two individuals homozygous for a 32 base-pair (bp) deletion in the CCR5 gene (CCR5Δ32 null) (Liu *et al.,* 1996; Samson *et al.,* 1996). T cells from these individuals fail to present CCR5 on the cell surface (Samson *et al.,* 1996); thus treatment with an R5 envelope should mediate effects exclusively through the CD4 receptor. Cells were treated with either 92MW959 gp120 (R5) or a second CCR5-specific envelope, JR-FL gp120 (O'Brien *et al.,* 1990). Sixty-five hours after envelopes were added, we observed increases in the number of TUNEL positive T cells in cultures treated with either R5-specific envelope (Figs. 2B, 2C, 2E, and 2F). In addition, if an R5 envelope was preincubated with a molar excess of sCD4, this effect was abrogated, further confirming that the apoptosis we observed in these individuals is dependent upon envelope–CD4 interactions. We conclude that envelope-mediated apoptosis can occur in the absence of both gp120-CCR5 and gp120-CXCR4 engagement.

Soluble CD4 enhances X4 gp120-mediated apoptosis in T lymphocytes

The data presented thus far indicate that although envelope–CD4 interactions are sufficient to induce apoptosis, interactions with HIV coreceptors, particularly CXCR4, may enhance this phenomenon. We next asked whether envelope–CXCR4 interactions in the absence of CD4 signaling are sufficient to mediate apoptosis. For most X4-specific envelopes, including 92Ug21-9, efficient engagement of CXCR4 is dependent upon an initial interaction with CD4 (Salzwedel *et al.,* 2000). This engagement leads to conformational changes in gp120 that increase its apparent affinity for CXCR4 (Lapham *et al.,* 1996). To eliminate envelope signaling through CD4 while achieving the necessary change in conformation of envelope, we preincubated 92Ug21-9 gp120 with a molar excess of sCD4.

Treatment of PBMCs with these complexes was compared to treatment with 92Ug21-9 gp120 alone. In the two donors we tested, addition of gp120–sCD4 complexes (Figs. 3C and 3G) resulted in a higher level of apoptosis than addition of gp120 alone (Figs. 3B and 3F). As expected, the addition of sCD4 alone had no measurable effect on apoptosis (Figs. 3D and 3H). We conclude that the enhanced activity of 92Ug21-9 gp120 in the presence of sCD4 resulted from the direct interaction of 92Ug21-9 gp120 with CXCR4.

Envelope–coreceptor interactions enhance gp120-mediated apoptosis

We next asked whether envelope signaling through CCR5 enhances envelope-mediated apoptosis. To address this possibility, we compared the capacity of R5 and X4 envelopes to induce apoptosis in $CD4^+/CCR5^+$ T cells. Freshly isolated $CD4^+/CCR5^+$ T cells constitute a relatively small fraction of freshly isolated PBMCs, ranging from 3 to 7% of the total T-lymphocyte population (data not shown). All of these cells express CXCR4. PBMCs from five individuals were treated with an R5 or an X4 envelope and then analyzed for TUNEL positivity after 65 h. Using mAb markers to identify the $CCR5^+$ subset of T lymphocytes, we observed that the R5-specific envelope induced a higher level of apoptosis in this subpopulation than the X4 envelope in four of the five donors tested (Fig. 4). Donor 1 failed to respond to either envelope. These results suggest that CCR5 signaling contributes to envelope-mediated apoptosis.

DISCUSSION

In the present study we have addressed the role of CD4, CXCR4, and CCR5 in envelope-mediated apoptosis of PBMCs. We compared the capacity of X4 and R5 envelopes to induce apoptosis in activated PBMC cultures. To determine whether CD4–envelope interactions, in the absence of coreceptor engagement, contribute to apoptosis, we treated PBMCs from an individual homozygous for a 32-bp deletion in CCR5 (CCR5Δ32 null) with an R5-specific envelope. R5 envelopes were capable of inducing apoptosis in these cells, indicating that coreceptor interactions are not an absolute requirement for envelope-induced apoptosis. In contrast, complexes of the R5 envelope and sCD4 failed to induce apoptosis in these PBMCs. These observations are consistent with reports of anti-CD4 mAb-induced apoptosis of $CD4^+$ T cells (Berndt *et al.,* 1998; Finkel *et al.,* 1995). Of note this result suggests that the capacity of an envelope to induce apoptosis is not restricted by the expression of a particular coreceptor. Thus, envelopes, via their ability to

FIG. 1. Comparison of apoptosis induced by an R5 and an X4 envelope in two donors. (A–E) Annexin-V and propidium iodide staining of anti-CD3/IL2 stimulated PBMCs after 5 h of treatment at 37°C. Light scatter of cells included for analysis is presented (A). Untreated PBMCs are presented for comparison (B), as are positive controls using the anti-FAS mAb CH11 (E). Treatment with the X4 envelope 92UG21-9 gp120 (C), and the R5 envelope 92MW959 gp120 (D) are presented. Percentage of annexin-V positive/propidium iodide negative cells appear in the lower right quadrant of each plot. (F–J) TUNEL staining of anti-CD3/IL2 stimulated PBMCs from a donor after 65 h of treatment at 37°C. Light scatter of cells included for analysis is presented (F). Untreated PBMCs are presented for comparison (G) as are positive controls using the anti-FAS mAb CH11 (J). Treatment with the X4 envelope 92UG21-9 gp120 (H), and with the R5 envelope 92MW959 gp120 (I). Percentage of TUNEL positive T cells, excluding the CD8-bright cells, are presented in the lower right quadrant of each plot, and percentage of CD8-bright, TUNEL positive cells are presented in the top right quadrant. These results are representative of data obtained from six independent experiments utilizing different donor PBMCs for both annexin-V and TUNEL assays.

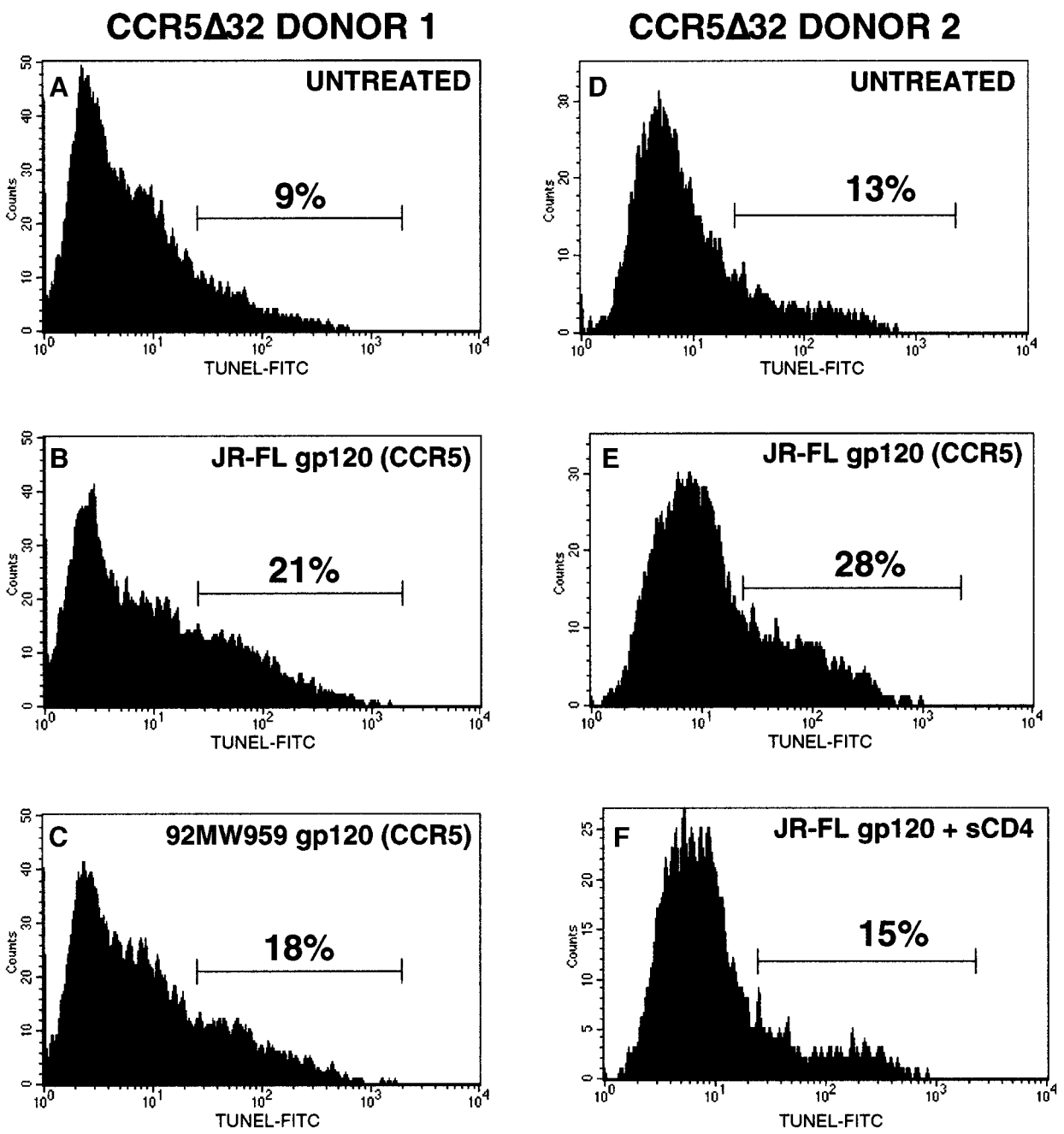

FIG. 2. Flow cytometric analysis of R5 envelope induced apoptosis in CCR5Δ32 homozygous T lymphocytes. T lymphocytes activated with anti-CD3/IL2 were treated with envelope protein at 37°C for 65 h and stained for TUNEL reactivity. The figure shows CCR5Δ32 homozygous T lymphocytes from two donors, untreated (A and D), treated with the R5 envelope JR-FL gp120 (B and E), or the R5 envelope 92MW959 gp120 (C). Histograms are gated on $CD3^+$ T cells. Percentage of TUNEL positive cells is presented above the boundary marker of each histogram. F demonstrates that sCD4 abrogated JR-FL envelope-mediated apoptosis.

interact with CD4, carry the potential to mediate apoptosis in microenvironments where the corresponding coreceptor required for viral entry and replication is absent.

Our analysis demonstrated that the X4 envelope induced higher levels of apoptosis. To assess the role of CXCR4 in the apoptotic response of T lymphocytes to an X4 envelope, we prepared complexes of an X4-specific envelope and sCD4. Such complexes should interact directly with CXCR4 but not with cell surface-bound CD4. Of note, gp120–sCD4 complexes induced higher levels of apoptosis in T lymphocytes than gp120 alone. This result demonstrates directly that envelope interactions with CXCR4 induces apoptosis and is consistent with previous studies (Berndt *et al.,* 1998; Biard-Piechaczyk *et al.,* 2000).

The enhancement of X4 envelope-induced apoptosis by sCD4 involves the induction of conformational changes in the envelope that allow optimal interaction

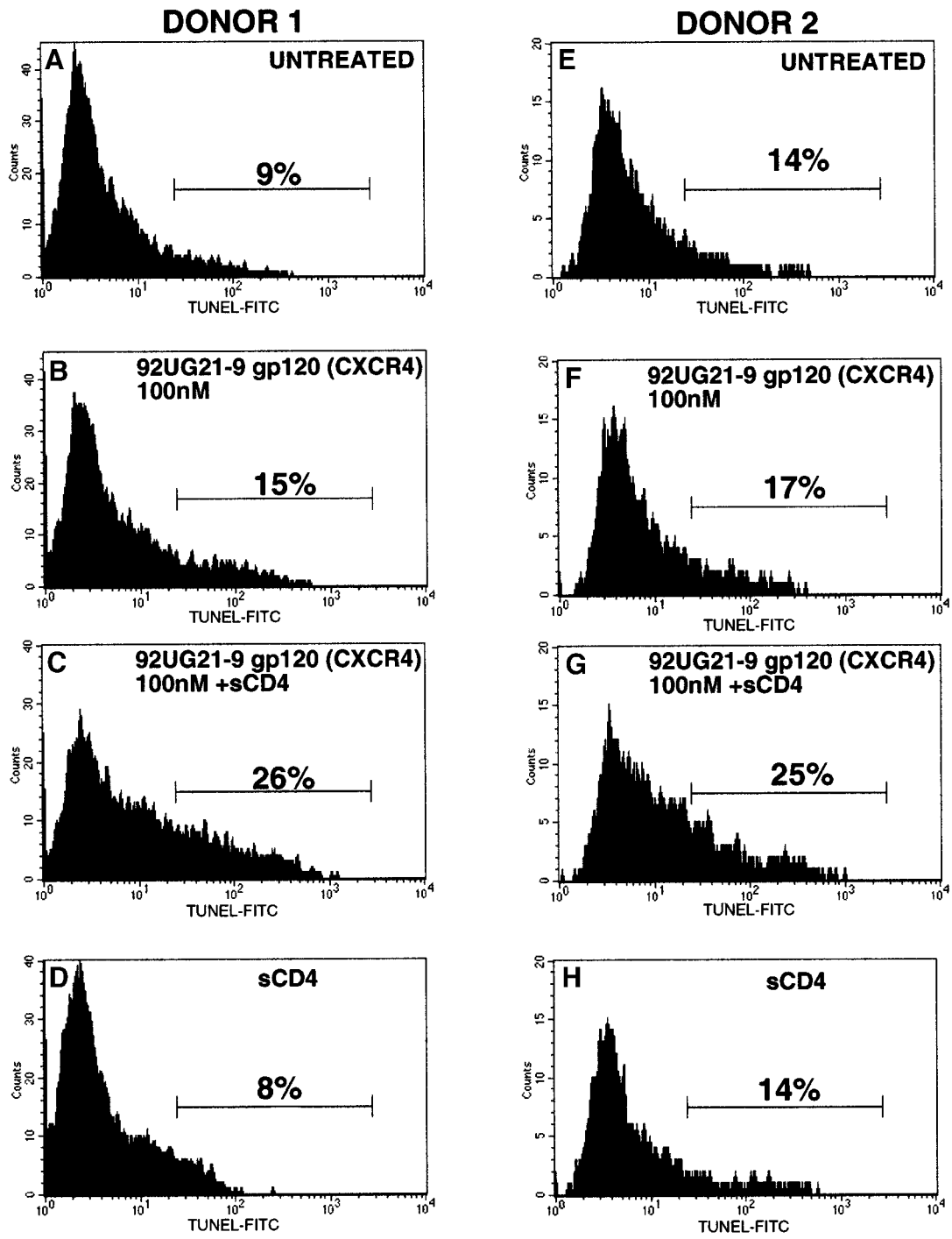

FIG. 3. Flow cytometric analysis of apoptosis induction in T lymphocytes by an X4 envelope in the presence or absence of sCD4. T lymphocytes activated with anti-CD3/IL2 were treated with envelope protein at 37°C for 65 h and stained for TUNEL reactivity. The figure shows untreated (A, E), treated with 92Ug21-9 (B, F) alone, 92Ug21-9 and sCD4 (C, G), or sCD4 alone (D, H). Histograms are gated on $CD3^+$ T cells. Percentage of TUNEL positive cells is presented above the boundary marker in each histogram. These results are representative of data obtained from six independent experiments utilizing different donor PBMCs.

with CXCR4 (Lapham *et al.,* 1996; Salzwedel *et al.,* 2000). The *in vivo* equivalent of this phenomenon likely relates the relationship between CD4 and CXCR4 on the cell membrane. Indeed, several reports suggest that CD4 is spatially segregated from CXCR4 on the plasma membrane (Lapham, 1996 No. 100; Xiao, 1999 No. 61) and that CD4 preferentially associates with other chemokine receptors including CCR5 (Xiao *et al.,* 1999). In this regard, the extent to which CD4 and CXCR4 are associated with each other on various T cell subsets may strongly influence the susceptibility of those subsets to envelope-induced apoptosis.

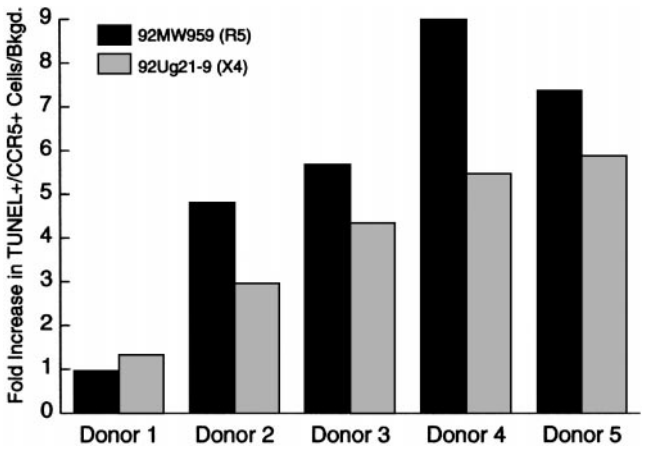

FIG. 4. Comparison of R5 and X4 envelope induced apoptosis in $CCR5^+$ T lymphocytes. Analysis of TUNEL results after treatment with the R5 envelope 92MW959 gp120 and the X4 envelope 92Ug21-9 gp120 induced apoptosis from five donor PBMCs. T lymphocytes activated with anti-CD3/IL2 were treated with envelope protein at 37°C for 65 h and stained for TUNEL reactivity. Analysis was carried out on the $CCR5^+$ lymphocyte-positive gate. Data represents the percentage increase in TUNEL reactivity in the $CCR5^+$ subpopulation of T lymphocytes relative to untreated cells of the same phenotype.

Having demonstrated that both CXCR4 and CD4 can play an active role in envelope-induced apoptosis, we addressed the role of CCR5 in this process. Addressing this question directly is made difficult by the fact that virtually all $CCR5^+$ T cells also express CXCR4. Thus, upon comparing the effects of an R5 vs an X4 envelope on $CCR5^+$ T cells, it is difficult to exclude interactions between the X4 envelope and CXCR4. Nevertheless, we found that, when whole PBMCs were treated, the subset of this population that expressed detectable levels of CCR5 was more susceptible to an R5 envelope than to an X4 envelope. Although the difference was modest, it is striking when compared to our previous result that included all T cells (Fig. 1), the majority of which do not express CCR5. In this population the X4 envelope was consistently more effective in inducing apoptosis.

In previous studies we and others have shown that signaling through CCR5 enhances viral replication (Arthos *et al.,* 2000; Kelly *et al.,* 1998; Kinter *et al.,* 1998). In this article we have demonstrated that envelope–CD4 engagement alone is sufficient to mediate apoptosis. We have further demonstrated that envelope–CCR5 and envelope–CXCR4 interactions can contribute to envelope-mediated apoptosis of activated PBMCs. For the latter however this contribution is limited by the degree of coassociation between CD4 and CXCR4. Thus, although the coreceptor specificity of a viral envelope may contribute to apoptosis, it is not the sole determinant. These conclusions underscore the potential for envelopes to induce cytopathic effects independent of their coreceptor specificity.

MATERIALS AND METHODS

Cells and reagents

For TUNEL and annexin-V staining, PBMCs were freshly isolated from individuals by Ficoll–Hypaque centrifugation. Cells from normal donors were determined to be homozygous for the wild-type CCR5 allele by PCR as previously described (Liu *et al.,* 1996). Freshly isolated cells were cultured in RPMI/10% FBS and activated with anti-CD3 (OKT3) (1 μg/ml) and IL-2 (100 u/ml) for 3 days prior to envelope or antibody treatment. The antibodies used for flow cytometry stainings, anti-CCR5, anti-CXCR4, anti-CD4, anti-CD8, were purchased from Pharmingen (San Diego, CA). We constructed, expressed, and purified all envelope proteins in Chinese hamster ovary (CHO) in an identical manner as previously described (Arthos *et al.,* 2000; Mossman *et al.,* 1996; Trkola *et al.,* 1996; Weissman *et al.,* 1997). Envelope proteins were determined to be endotoxin-free using the chromogenic limulus amebocyte lysate method (BioWhittaker, Walkersville, MD). CHO cell lines expressing these recombinant envelopes have been provided to the AIDS Reference and Reagent Program (www.aidsreagent.org). Envelopes utilized included those derived from HIV-1 JRFL, a CCR5-utilizing molecular clone (O'Brien *et al.,* 1990); HIV-1 NL43, a tissue culture adapted CXCR4-utilizing molecular clone (Adachi *et al.,* 1986); HIV-1 92Ug21-9, a primary CXCR4-utilizing biological clone (Gao *et al.,* 1994); and HIV-1 92MW959, a CCR5-utilizing biological clone (Gao *et al.,* 1994). Coreceptor utilization is described in "Coreceptor Use by Primate Lentiviruses" url: hiv-web.lanl.gov/REVIEWS/BRODER99/coreceptor.html. In addition 92Ug21-9 is a CXCRX4-specific envelope as described in Genbank submission, Accession No. U08804. Soluble CD4 (sCD4) (SmithKline Beecham, King of Prussia, PA) was used to generate envelope/sCD4 complexes. The anti-Fas mAb CH11 (Beckman-Coulter, Fullerton, CA) was used as a positive control for both annexin-V and TUNEL assays. Reference controls designated as untreated included an appropriate volume of a mock protein preparation derived and purified from the parental CHO cell line from which each of the gp120-expressing cell lines was derived.

Annexin V staining

Freshly isolated PBMCs (2×10^5 cells) were cultured in RPMI/10% FBS in 96-well plates. Cells were treated with HIV envelope (50 nM) for 5 h at 37°C and subsequently stained with annexin-V FITC and propidium iodide per manufacturers' instructions (R & D Technologies, Minneapolis, MN). The FAS mAb CH11 was used as a positive control to induce apoptosis. Untreated controls included a mock protein preparation. FAS-treated cells were used to define the population (by light scatter) of lymphocytes analyzed. We defined quadrants based

upon the annexin-V staining of the untreated population (negative control) such that greater than 97% of the cells fall within the lower left quadrant. For each donor we compared the light scatter of untreated cells vs cells treated with the anti-FAS mAb CH11. Upon treatment with CH11, a distinct population of cells appears. We draw our gate to include that population.

TUNEL assays

Freshly isolated PBMCs (2×10^5 cells) were cultured in RPMI/10% FBS in 96-well plates. Cells were treated with envelope (50–100 nM) for approximately 65 h at 37°C and with terminal deoxynucleotidyl transferase according to the manufacturer's instructions (Beckman-Coulter, Miami, FL). A lymphocyte gate was established using forward and side light scatter. For each donor we compared the light scatter of untreated cells vs cells treated with the anti-FAS mAb CH11. Upon treatment with CH11, a distinct population of cells appears. We draw our gate to include that population. Cells were stained with anti-CD3, anti-CD8, and FITC-labeled dUTP. The FAS mAb CH11 was used a positive control to induce apoptosis. Mock protein preparations were included in the untreated control.

REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**(2), 284–291.

Arthos, J., Rubbert, A., Rabin, R. L., Cicala, C., Machado, E., Wildt, K., Hanbach, M., Steenbeke, T. D., Swofford, R., Farber, J. M., and Fauci, A. S. (2000). CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J. Virol.* **74**(14), 6418–6424.

Bacon, K. B., Szabo, M. C., Yssel, H., Bolen, J. B., and Schall, T. J. (1996). RANTES induces tyrosine kinase activity of stably complexed p125FAK and ZAP-70 in human T cells. *J. Exp. Med.* **184**(3), 873–882.

Badley, A. D., Dockrell, D., and Paya, C. V. (1997). Apoptosis in AIDS. *Adv. Pharmacol.* **41,** 271–294.

Berndt, C., Mopps, B., Angermuller, S., Gierschik, P., and Krammer, P. H. (1998). CXCR4 and CD4 mediate a rapid CD95-independent cell death in CD4(+) T cells. *Proc. Natl. Acad. Sci. USA* **95**(21), 12556–12561.

Biard-Piechaczyk, M., Robert-Hebmann, V., Richard, V., Roland, J., Hipskind, R. A., and Devaux, C. (2000). Caspase-dependent apoptosis of cells expressing the chemokine receptor CXCR4 is induced by cell membrane-associated human immunodeficiency virus type 1 envelope glycoprotein (gp120). *Virology* **268**(2), 329–344.

Biberfeld, P., Chayt, K. J., Marselle, L. M., Biberfeld, G., Gallo, R. C., and Harper, M. E. (1986). HTLV-III expression in infected lymph nodes and relevance to pathogenesis of lymphadenopathy. *Am. J. Pathol.* **125**(3), 436–442.

Blanco, J., Jacotot, E., Cabrera, C., Cardona, A., Clotet, B., De Clercq, E., and Este, J. A. (1999). The implication of the chemokine receptor CXCR4 in HIV-1 envelope protein-induced apoptosis is independent of the G protein-mediated signalling. *AIDS* **13**(8), 909–917.

Bossy-Wetzel, E., and Green, D. R. (2000). Detection of apoptosis by annexin V labeling. *Methods Enzymol.* **322,** 15–18.

Carbonari, M., Cibati, M., and Fiorilli, M. (1995). Measurement of apoptotic cells in peripheral blood. *Cytometry* **22**(3), 161–167.

Cheng-Mayer, C., Seto, D., Tateno, M., and Levy, J. A. (1988). Biologic features of HIV-1 that correlate with virulence in the host. *Science* **240**(4848), 80–82.

Chirmule, N., and Pahwa, S. (1996). Envelope glycoproteins of human immunodeficiency virus type 1: profound influences on immune functions. *Microbiol. Rev.* **60**(2), 386–406.

Cicala, C., Arthos, J., Rubbert, A., Selig, S., Wildt, K., Cohen, O. J., and Fauci, A. S. (2000). HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4+ T cells. *Proc. Natl. Acad. Sci. USA* **97**(3), 1178–1183.

Cicala, C., Arthos, J., Ruiz, M., Vaccarezza, M., Rubbert, A., Riva, A., Wildt, K., Cohen, O., and Fauci, A. S. (1999). Induction of phosphorylation and intracellular association of CC chemokine receptor 5 and focal adhesion kinase in primary human CD4+ T cells by macrophage-tropic HIV envelope. *J. Immunol.* **163**(1), 420–426.

Colamussi, M. L., Secchiero, P., Zella, D., Curreli, S., Mirandola, P., Capitani, S., and Zauli, G. (2000). Stromal derived factor-1 alpha induces apoptosis in activated primary CD4+ T cells [letter]. *AIDS* **14**(6), 748–750.

Davis, C. B., Dikic, I., Unutmaz, D., Hill, C. M., Arthos, J., Siani, M. A., Thompson, D. A., Schlessinger, J., and Littman, D. R. (1997). Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J. Exp. Med.* **186**(10), 1793–1798.

Fauci, A. S. (1996). Host factors and the pathogenesis of HIV-induced disease. *Nature* **384**(6609), 529–534.

Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Baba, T. W., Ruprecht, R. M., and Kupfer, A. (1995). Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes [see comments]. *Nat. Med.* **1**(2), 129–134.

Ganju, R. K., Dutt, P., Wu, L., Newman, W., Avraham, H., Avraham, S., and Groopman, J. E. (1998). Beta-chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood* **91**(3), 791–797.

Gao, F., Yue, L., Craig, S., Thornton, C. L., Robertson, D. L., McCutchan, F. E., Bradac, J. A., Sharp, P. M., and Hahn, B. H. (1994). Genetic variation of HIV type 1 in four World Health Organization-sponsored vaccine evaluation sites: Generation of functional envelope (glycoprotein 160) clones representative of sequence subtypes A, B, C, and E. WHO Network for HIV Isolation and Characterization. *AIDS Res. Hum. Retroviruses* **10**(11), 1359–1368.

Grivel, J. C., Malkevitch, N., and Margolis, L. (2000a). Human immunodeficiency virus type 1 induces apoptosis in CD4+ but not in CD8+ T cells in Ex vivo-infected human lymphoid tissue. *J. Virol.* **74**(17), 8077–8084.

Grivel, J. C., and Margolis, L. B. (1999). CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue [see comments]. *Nat. Med.* **5**(3), 344–346.

Grivel, J. C., Penn, M. L., Eckstein, D. A., Schramm, B., Speck, R. F., Abbey, N. W., Herndier, B., Margolis, L., and Goldsmith, M. A. (2000b). Human immunodeficiency virus type 1 coreceptor preferences determine target T-cell depletion and cellular tropism in human lymphoid tissue. *J. Virol.* **74**(11), 5347–5351.

Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A., and Ameisen, J. C. (1992). Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J. Exp. Med.* **175**(2), 331–340.

Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., O'Brien, W. A., and Verdin, E. (1998). Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4 [see comments]. *Nature* **395**(6698), 189–194.

Hesselgesser, J., Taub, D., Baskar, P., Greenberg, M., Hoxie, J., Kolson, D. L., and Horuk, R. (1998). Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. *Curr. Biol.* **8**(10), 595–598.

Hivroz, C., Mazerolles, F., Soula, M., Fagard, R., Graton, S., Meloche, S., Sekaly, R. P., and Fischer, A. (1993). Human immunodeficiency virus

gp120 and derived peptides activate protein tyrosine kinase p56lck in human CD4 T lymphocytes. *Eur. J. Immunol.* **23**(3), 600–607.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection [see comments]. *Nature* **373**(6510), 123–126.

Kaul, M., and Lipton, S. A. (1999). Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. *Proc. Natl. Acad. Sci. USA* **96**(14), 8212–8216.

Kelly, M. D., Naif, H. M., Adams, S. L., Cunningham, A. L., and Lloyd, A. R. (1998). Dichotomous effects of beta-chemokines on HIV replication in monocytes and monocyte-derived macrophages. *J. Immunol.* **160**(7), 3091–3095.

Kinter, A., Catanzaro, A., Monaco, J., Ruiz, M., Justement, J., Moir, S., Arthos, J., Oliva, A., Ehler, L., Mizell, S., Jackson, R., Ostrowski, M., Hoxie, J., Offord, R., and Fauci, A. S. (1998). CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4+ T cells: Role of signal transduction. *Proc. Natl. Acad. Sci. USA* **95**(20), 11880–11885.

Lapham, C. K., Ouyang, J., Chandrasekhar, B., Nguyen, N. Y., Dimitrov, D. S., and Golding, H. (1996). Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines [see comments]. *Science* **274**(5287), 602–605.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**(3), 367–377.

McCloskey, T. W., Ott, M., Tribble, E., Khan, S. A., Teichberg, S., Paul, M. O., Pahwa, S., Verdin, E., and Chirmule, N. (1997). Dual role of HIV Tat in regulation of apoptosis in T cells. *J. Immunol.* **158**(2), 1014–1019.

Monte, D., Groux, H., Raharinivo, B., Plouvier, B., Dewulf, J., Clavel, T., Grangette, C., Torpier, G., Auriault, C., Capron, A., *et al.* (1992). Productive human immunodeficiency virus-1 infection of megakaryocytic cells is enhanced by tumor necrosis factor-alpha. *Blood* **79**(10), 2670–2679.

Moore, J. P., Trkola, A., and Dragic, T. (1997). Co-receptors for HIV-1 entry [see comments]. *Curr. Opin. Immunol.* **9**(4), 551–562.

Mossman, S. P., Bex, F., Berglund, P., Arthos, J., O'Neil, S. P., Riley, D., Maul, D. H., Bruck, C., Momin, P., Burny, A., Fultz, P. N., Mullins, J. I., Liljestrom, P., and Hoover, E. A. (1996). Protection against lethal simian immunodeficiency virus SIVsmmPBj14 disease by a recombinant Semliki Forest virus gp160 vaccine and by a gp120 subunit vaccine. *J. Virol.* **70**(3), 1953–1960.

O'Brien, W. A., Koyanagi, Y., Namazie, A., Zhao, J. Q., Diagne, A., Idler, K., Zack, J. A., and Chen, I. S. (1990). HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature* **348**(6296), 69–73.

Ohagen, A., Ghosh, S., He, J., Huang, K., Chen, Y., Yuan, M., Osathanondh, R., Gartner, S., Shi, B., Shaw, G., and Gabuzda, D. (1999). Apoptosis induced by infection of primary brain cultures with diverse human immunodeficiency virus type 1 isolates: Evidence for a role of the envelope. *J. Virol.* **73**(2), 897–906.

Popik, W., Hesselgesser, J. E., and Pitha, P. M. (1998). Binding of human immunodeficiency virus type 1 to CD4 and CXCR4 receptors differentially regulates expression of inflammatory genes and activates the MEK/ERK signaling pathway. *J. Virol.* **72**(8), 6406–6413.

Salzwedel, K., Smith, E. D., Dey, B., and Berger, E. A. (2000). Sequential CD4-coreceptor interactions in human immunodeficiency virus type 1 Env function: Soluble CD4 activates Env for coreceptor-dependent fusion and reveals blocking activities of antibodies against cryptic conserved epitopes on gp120. *J. Virol.* **74**(1), 326–333.

Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G., and Parmentier, M. (1996). Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene [see comments]. *Nature* **382**(6593), 722–725.

Stewart, S. A., Poon, B., Song, J. Y., and Chen, I. S. (2000). Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. *J. Virol.* **74**(7), 3105–3111.

Sunila, I., Vaccarezza, M., Pantaleo, G., Fauci, A. S., and Orenstein, J. M. (1997). gp120 is present on the plasma membrane of apoptotic CD4 cells prepared from lymph nodes of HIV-1-infected individuals: An immunoelectron microscopic study. *AIDS* **11**(1), 27–32.

Tamma, S. M., Chirmule, N., Yagura, H., Oyaizu, N., Kalyanaraman, V., and Pahwa, S. (1997). CD4 cross-linking (CD4XL) induces RAS activation and tumor necrosis factor-alpha secretion in CD4+ T cells. *Blood* **90**(4), 1588–1593.

Tersmette, M., Gruters, R. A., de Wolf, F., de Goede, R. E., Lange, J. M., Schellekens, P. T., Goudsmit, J., Huisman, H. G., and Miedema, F. (1989a). Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: Studies on sequential HIV isolates. *J. Virol.* **63**(5), 2118–2125.

Tersmette, M., Lange, J. M., de Goede, R. E., de Wolf, F., Eeftink-Schattenkerk, J. K., Schellekens, P. T., Coutinho, R. A., Huisman, J. G., Goudsmit, J., and Miedema, F. (1989b). Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. *Lancet* **1**(8645), 983–985.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5 [see comments]. *Nature* **384**(6605), 184–187.

Ullrich, C. K., Groopman, J. E., and Ganju, R. K. (2000). HIV-1 gp120- and gp160-induced apoptosis in cultured endothelial cells is mediated by caspases. *Blood* **96**(4), 1438–1442.

van't Wout, A. B., Kootstra, N. A., Mulder-Kampinga, G. A., Albrecht-van Lent, N., Scherpbier, H. J., Veenstra, J., Boer, K., Coutinho, R. A., Miedema, F., and Schuitemaker, H. (1994). Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J. Clin. Invest.* **94**(5), 2060–2067.

Weissman, D., Rabin, R. L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J. M., and Fauci, A. S. (1997). Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* **389**(6654), 981–985.

Xiao, X., Wu, L., Stantchev, T. S., Feng, Y. R., Ugolini, S., Chen, H., Shen, Z., Riley, J. L., Broder, C. C., Sattentau, Q. J., and Dimitrov, D. S. (1999). Constitutive cell surface association between CD4 and CCR5. *Proc. Natl. Acad. Sci. USA* **96**(13), 7496–7501.

Zheng, J., Ghorpade, A., Niemann, D., Cotter, R. L., Thylin, M. R., Epstein, L., Swartz, J. M., Shepard, R. B., Liu, X., Nukuna, A., and Gendelman, H. E. (1999). Lymphotropic virions affect chemokine receptor-mediated neural signaling and apoptosis: Implications for human immunodeficiency virus type 1-associated dementia. *J. Virol.* **73**(10), 8256–8267.